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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The first year of the research performed under Award Number DAMD17-02-1-0206 has yielded promising results with respect to the application of MMP inhibitor BB94 on HD- exposed human skin pieces in order to prevent microvesication. BB94 is effective at a concentration of at least 1 μg/ml and can be added to the culture medium as late as 8 h after exposure to HD, without loosing its inhibiting effect on epidermal-dermal separation. From a therapeutic angle, this time window might allow non-urgent cure of HD casualties.

In an adjacent series of experiments, application of proteomic analysis as an unbiased way to study cellular events following HD-exposure has proven to be a valuable approach. 2D-PAGE analysis of the protein profile of cultured HEK that were exposed to 100 μM HD gave clues for the induction of apoptosis by the gradual appearance over 24 h of fragments of the type I keratins K14 and K17, generated by caspase cleavage. It is expected that the identification of several other new spots that appeared in the protein pattern of HD-exposed HEK might shed more light on the mechanistic toxicology of HD.

The results obtained in this study so far, suggest a dual treatment of HD-exposed skin: application of a MMP-inhibitor to prevent microvesication together with the use of a caspase-inhibitor to reduce epidermal cell damage.

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List of abbreviations

CHAPS	3-[3-Cholamidopropyl)-dimethyl-ammonio]-1-propane sulfonate
2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
DNase	Deoxyribonuclease
DTT	1,4-Dithiotreitol
HD	Sulfur mustard
HEK	Human epidermal keratinocytes
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
kDa	Kilodalton
KGM	Keratinocyte growth medium
KBM	Keratinocyte basal medium
MALDI-TOF	Matrix- assisted laser desorption/ionization – time of flight
μM	Micromolar
mM	Millimolar
MMP	Matrix metalloproteinase
MS	Mass spectrometry
MW	Molecular weight
PBS	Phosphate buffered saline
pI	Isoelectric point
RCDC	Reductant compatible, detergent compatible
RNase	Ribonuclease
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Tris	Tris(hydroxymethyl)-amino-methane

1 Introduction

The studies described in this report are part of a large research project (award number DAMD17-02-1-0206) aimed to obtain adequate medical defense against injury caused by exposure to sulfur mustard (HD). The project will elucidate the mechanism behind epidermal-dermal separation in HD-exposed human skin in order to bring a causal therapy against blister formation. Loss of the attachment competence of the basement membrane proteins, including the hemidesmosomal proteins, is postulated to be a specific cause of HD-induced vesication of the skin and a disturbed balance between production and degradation of these proteins has been suggested contributory. It is known that basement membrane and hemidesmosomal proteins are specifically degraded by matrix metalloproteinases (MMPs), a family of enzymes that is involved in normal turnover of the extracellular matrix (Kleiner and Stetler-Stevenson, 1999; Nagase and Woessner, 1999). In the past year, we have investigated various aspects of the therapeutic potential of the synthetic MMP inhibitor BB94, the first of a series of compounds that act either directly or indirectly as inhibitors of MMPs. These investigations were performed conform Task 1 of the statement of work mentioned in the research grant proposal.

However, when following such an approach, i.e., based on an existing hypothesis, certain events may remain unrecognized as key factors in the mechanism of HD-induced vesication. Therefore, we applied proteome analysis as a global approach to reveal unexpected cellular events occurring after HD exposure. Perturbations in protein expression of cultured human epidermal keratinocytes (HEK) were examined by comparison of protein expression profiles and identification of differentially expressed proteins in control and HD-treated samples. A comparative analysis has been made of protein profiles expressed in HEK at 8, 18 and 24 h following exposure to 0 or 100 μM HD. These investigations were performed conform Task 5 of the statement of work mentioned in the research grant proposal.

The studies that are described in this report are not in full accordance with the goals that were set for the first year in the statement of work. Due to lack of personnel, only limited studies were performed. The delay in work will be compensated for in the second year.

2 Experimental Methods

2.1 HD synthesis

HD has been synthesized at TNO-Prins Maurits Laboratory and has a purity of at least 97%.

2.2 Exposure of skin pieces to HD vapor and human skin organ culture

Human mammary skin was obtained from cosmetic surgery with informed consent of the patient.

Human mammary skin has been exposed to saturated HD vapor at 25 °C for various periods of time using a vapor cup device as described earlier (Mol et al., 1991). Organ cultures of human skin were maintained as described by Varani et al., (1995). Skin pieces of 0.25 cm^2 were floated with the dermal side down in keratinocyte basal medium (BioWhittaker Europe, Verviers, Belgium) supplemented with $CaCl_2$ to a final concentration of 1.4 mM (KBMCa; 1 ml medium/well of a 12 well cluster plate) and incubated at 37 °C in an atmosphere of 6% CO_2 in air for 48 h.

The MMP inhibitor BB94 (a gift of British Biotech, Oxford, UK) was dissolved in DMSO and added to the organ culture medium in concentrations ranging from 0.1 to 5.0 μg/ml. The final concentration of DMSO was 1%.

2.3 Histology

Human skin pieces were fixed overnight at 4 °C in 2% paraformaldehyde in phosphate buffered saline (PBS). They were stored in 70% ethanol until embedding in paraffin. Sections were stained with hematoxylin/eosin and examined by light microscope.

2.4 Human epidermal keratinocyte (HEK) culture and exposure of HEK to HD

First passage HEK was grown confluent in serum-free keratinocyte growth medium (KGM) (BioWhittaker Europe, Verviers, Belgium). To study the cellular response of the cells to HD, the cells were exposed for 30 min to 0 or 100 μM HD.

One day after reaching confluence, HEK were exposed to HD (2.5 ml/well of a 6-well cluster plate) for 30 min at 25 °C. Stock solutions of HD were freshly prepared in dry acetone and diluted immediately before use in KBM to obtain the desired working concentrations. The final concentration of acetone in the incubation medium was 1%.

The cells were washed and processed either immediately or after an incubation period at 37 °C in KGM. Cell lysates prepared for two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) following 8, 18 and 24 h of post-exposure incubation with fresh KGM. Lysates of three wells with identical treatment were pooled.

This experiment was repeated twice, on the understanding that in one experiment lysates were collected only at 24 h after HD exposure.

2.5 2D-PAGE

Cells were solubilized in 40 mM Tris buffer (pH 8.0) containing 0.5% SDS, 100 mM DTT, protease inhibitor cocktail (Complete ® Mini, Roche Diagnostics, Mannheim, Germany) and DNase I/ RNaseA (Roche Diagnostics, Mannheim, Germany). For removal of interfering contaminations, proteins were precipitated in 11% trichloroacetic acid in ice-cold acetone supplemented with 25 mM DTT. Pellets were washed twice with ice-cold acetone and subsequently dissolved in rehydration buffer consisting of 9.5 M urea, 4% CHAPS, 0.5% IPG buffer and 25 mM DTT. Protein was quantified by using a RCDC protein assay kit (BioRad, Veenendaal, The Netherlands). Samples of 100 – 300 μg of protein were loaded on 13 cm Immobiline IPG strips (4-7) (Amersham Bioscience Biotech, Roosendaal, The Netherlands). Rehydration was performed on an IPGphor apparatus under low voltage (6 h, 30V; 6h, 60 V) for improved sample entry. Next, isoelectric focusing (IEF) was carried out at 20° C for a total of 34 kVh. Before SDS-PAGE, the focused IPG strips were equilibrated, reduced and alkylated in a buffer containing 6 M urea, 50 mM Tris-HCl, pH 8.8, 2% SDS, and 30 % glycerol with either 10 mg DTT/ml or 25 mg iodoacetamide/ml. Then, the proteins were separated in the second dimension on homogeneous polyacrylamide gels (10% T). The SDS-PAGE was run at a constant current of 30 mA/gel at 20°C. At least four 2D-gels of each lysate sample were produced. Gels were fixed overnight and stained by a mass spectrometry compatible silver staining (Yan et al., 2000). Gels were digitized using a flatbed scanner (Amersham Bioscience Biotech, Roosendaal, The Netherlands). Comparative qualitative analysis of the protein patterns has been performed partially by eye and partially by using ImageMaster 2D Elite software (Amersham Pharmacia Biotech, Roosendaal, The Netherlands). For each comparison at least four replicate gels of each sample were used. For changes in proteins spots to be considered as genuine differences in protein expression we applied the following criterion: they should show similar qualitative changes in expression in all examined gels originating from one sample.

2.6 Protein identification

For protein identification, spots of interest were excised and cut into 1-2 mm^3 pieces. The particles were washed, reduced and alkylated before they were digested with 12.5 ng/μl trypsin modified sequencing grade (Roche Diagnostics, Mannheim, Germany) in 5 mM $CaCl_2$

and 25 mM NH_4HCO_3 overnight at 37 °C on a shaker (Jensen et al., 1999). The peptides were extracted twice with 5% formic acid and acetonitrile (1:1) and the extracts were pooled and dried in a vacuum centrifuge. Before desalting, the peptides were dissolved in 5% formic acid. Desalting was performed with ZipTip microcolumns of C18 (Millipore, Etten-Leur, The Netherlands). Peptide mass fingerprints were determined by matrix assisted laser desorption ionization (MALDI)-time-of-flight (TOF)-MS on a Bruker Reflex III TOF mass spectrometer using α-cyano-4-hydroxycinnamic acid as the matrix. The mass spectra were internally calibrated with the autoproteolytic trypsin T4 fragment of 2163.06 Da. The resulting spectra gave lists of masses that were entered into the Profound (NCBI) program (www.. Other parameters were: allowance for two missed cuts by trypsin digestion, cysteine conversion into carboxyamidomethyl cysteine and the occurrence of oxidized methionine. Search criteria for protein identification were 0.1 Da mass accuracy and a match of at least four tryptic fragments. The ProFound score 'Z-value', indicating the quality of the database search results, had to be higher than 0.5. The SWISS-PROT database (http://www.expasy.ch/sprot/) was used for additional information on pI, MW and amino acid composition of the identified proteins.

3 Results

3.1 Therapeutic effect of MMP inhibitor BB94

Control human skin, cultured in KBMCa for 48 h has a normal appearance (*Figure 1A*). Exposure of human skin pieces to saturated vapor of HD at 25 °C for 5 min results in clear epidermal damage with pyknotic nuclei and in microvesication after a culture period of 48 h in KBMCa (*Figure 1B*). When BB94 was present in the culture medium, epidermal-dermal separation was prevented with concentrations of 1 μg/ml or higher (*Figure 1 D - 1 I*). The presence of the solvent DMSO (1%) had no attenuating effect on the lesion (*Figure 1C*).

Subsequently, it was investigated how much time might pass between HD exposure and the application of the inhibitor without loss of protective effect. In *Figure 2* it is shown that BB94 can be added as late as 8 h after exposure to HD, without loosing its inhibiting effect on epidermal-dermal separation. It is observed that the MMP inhibitor had no effect on the HD-induced cellular necrosis in the epidermis.

3.2 2D-PAGE of human epidermal keratinocytes

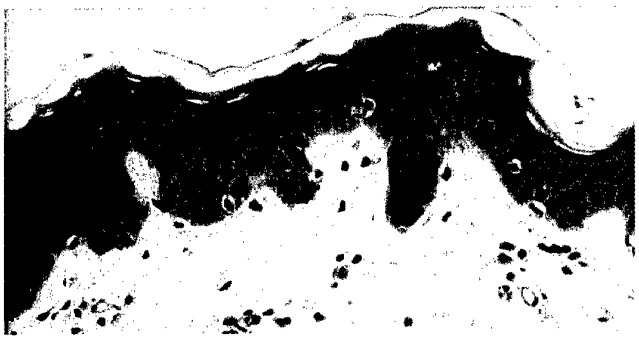
A typical image of the silver stained 2D protein expression pattern of control HEK is shown in *Figure 3.* Proteins were separated over an isoelectric point (pI) range of 4-7 and a molecular weight (MW) range of 15 - 200 kDa. To start a reference proteome map for cultured HEK, indicated spots were randomly selected and after digestion with trypsin, subjected to MALDI-TOF-MS peptide mass fingerprinting. So far, 22 proteins could be identified and the corresponding protein names are listed in *Table 1.* Theoretical MW and pI are given according to SWISS-PROT entries. Two large smears on the gel contain keratins 14 and 17. It is supposed that both keratins are present in such abundance in the keratinocyte lysate, that they cause smears at a distance from their actual location on the gel.

In our results, many of the identified proteins are located on the gel at sites that are in full agreement with theoretical values for their pI and MW. For several other of the identified proteins the observed location on the gel deviates slightly from their theoretical values for pI and MW. These shifts are probably attributed to post-translational modifications, such as glycosylation, phosphorylation or proteolytic cleavage.

3.3 Differential display of HEK proteins associated with exposure to HD

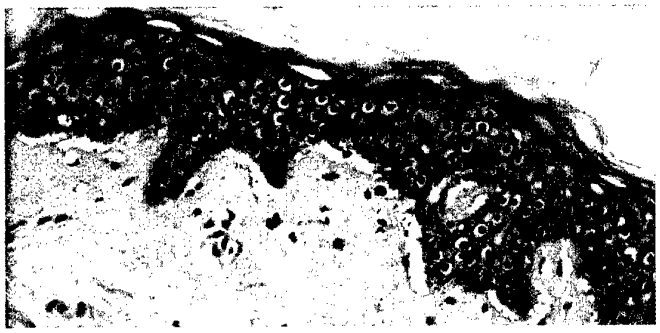
Comparative qualitative analysis of gels obtained at 8, 18 and 24 h of incubation following exposure to 0 or 100 μM HD demonstrated several alterations in protein expression that were associated with exposure to HD. As silver staining is not a quantitative method, only proteins spots that consistently showed a treatment-dependent change in intensity compared to control were referred to as HD-exposure related. Several new spots came up in the protein profile of HD-exposed HEK at 24 h after exposure. The most remarkable HD-exposure related changes in the protein pattern are seen in the area with pI 4.5 – 5.5 and MW 20 -30 kDa, indicated as a red box in *Figure 3*. Typical patterns in this area obtained in each of the three experiments performed are given in *Figure 4*. For each experiment the protein patterns of control and HD-treated HEK at 24 h post-exposure were compared. Newly appearing spots are indicated with arrows. The nine indicated protein spots A through I in *Figure 4* were observed as qualitative treatment-dependent alterations occurring in all examined gels of all three 24 h samples. The pattern at 24 h is the result of a gradually increase in spot intensities starting from 8 h post-incubation time (*Figure 5*).

So far, peptide mass fingerprinting has yielded the identities of three spots. Spots **G** and **A** are fragments of type I keratin 14 (K14). Peptide mass fingerprinting yielded eight tryptic fragments in spot **G** that matched with T3, T4, T5, T10, T15, T17, T18, and T23 in N-terminal part of the protein, whereas the three tryptic fragments that were observed in spot **A** were exclusively from the C-terminal part of K14 (T36, T40 and T46) (*Figure 6a*). Probably, spot **G** is an N-terminal fragment with a theoretical MW of 28.5 and pI of 5.1 and spot **A** is a C-terminal fragment with a theoretical MW of 22.9 and pI of 5.1. These two fragments of K14 are supposed to be generated by caspase-6 cleavage, which generally occurs under apoptotic conditions. The caspase-6 motif for cleavage is VEMD, indicated in green in *Figure 6a*. A simultaneous disappearance of full-length K14 could not be observed, as K14 is excessively present in the keratinocyte lysate (see above). Spot **C** is probably the C–terminal fragment of type I keratin 17 with a theoretical MW of 22.0 and pI of 5.0. The observed tryptic fragments are, with one exception, from the protein fragment that follows after the VEMD motif (*Figure 6b*). Identification of the other difference spots is under way.

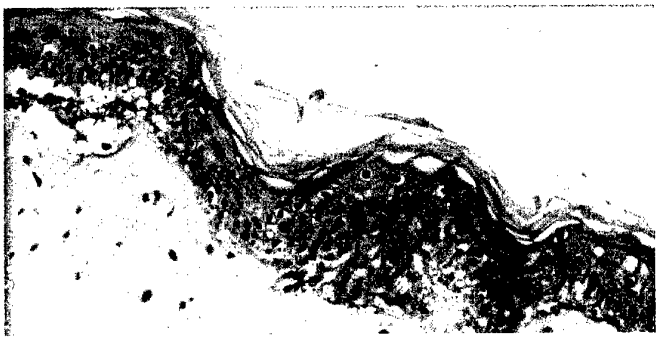


A. Control

B. HD/ no treatment



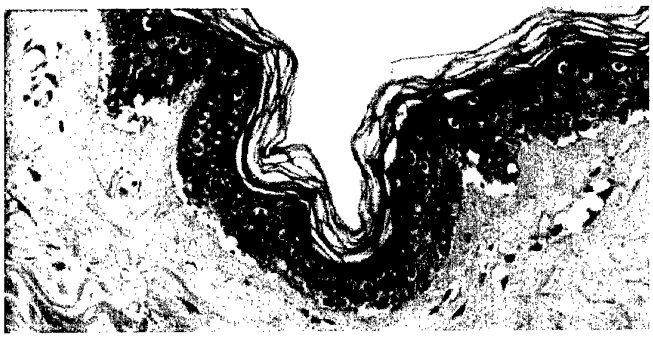
C. HD/1%DMSO/ 0 h



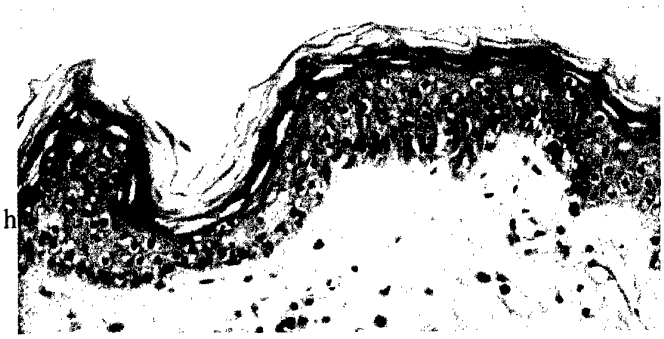
D. HD/0.1 μg BB94/ 0 h

E. HD/0.25 μg BB94/ 0 h

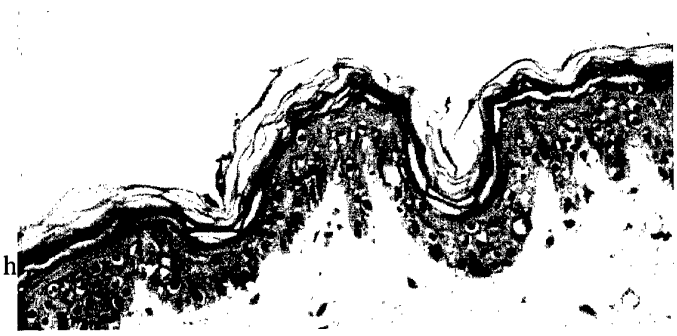
F. HD/0.5 μg BB94/ 0 h



G. HD/1.0 μg BB94/ 0 h

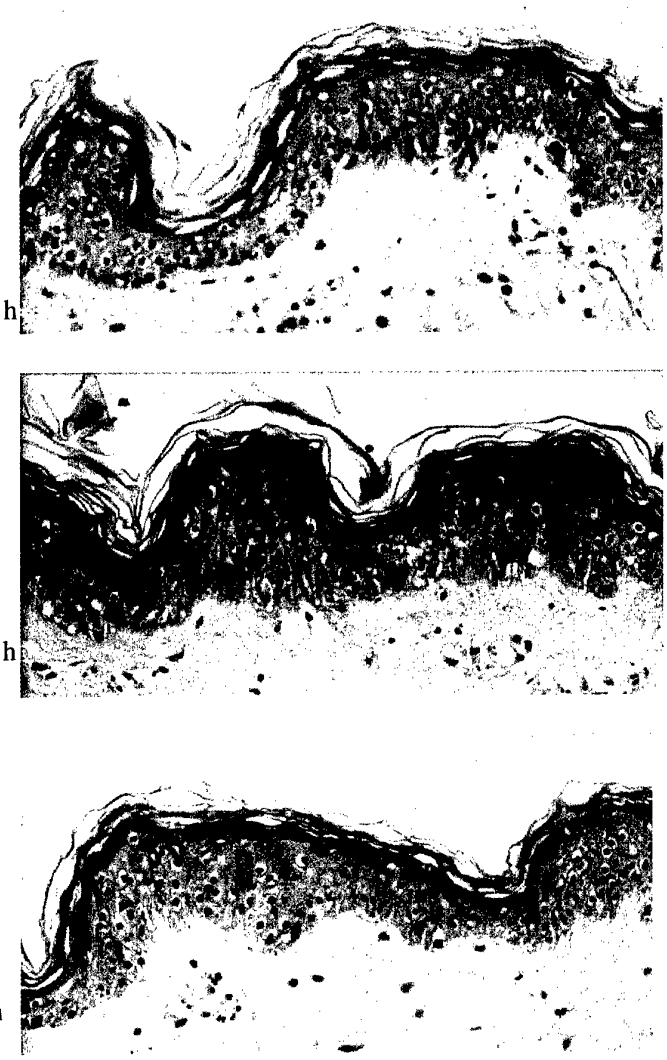


H. HD/2.0 μg BB94/ 0 h



I. HD/5.0 μg BB94/ 0 h

Figure 1. Effects of MMP-inhibitor BB94 on epidermal-dermal separation in human skin that has been exposed to saturated vapor of HD for 5 min. After exposure, skin is organ cultured in KBMCa for 48 h in the presence of various concentrations of the inhibitor (0 to 5.0 μg/ml) Epidermal-dermal separation is inhibited with concentrations of 1 μg/ml and higher.



A. HD/1.0 μg BB94/ 0 h

B. HD/1.0 μg BB94/ 4 h

C. HD/ 1.0 μg BB94/ 8 h

Figure 2. Effects of delayed application of MMP-inhibitor BB94 on epidermal-dermal separation in human skin that has been exposed to saturated vapor of HD for 5 min. After exposure, skin is organ cultured in KBMCa, to which 1.0 μg/ml BB94 was added immediately (A) and at 4 (B) and 8 h (C) after exposure. There is still a full protective effect of BB94 on epidermal-dermal separation, when BB94 is added at 8 h after HD exposure.

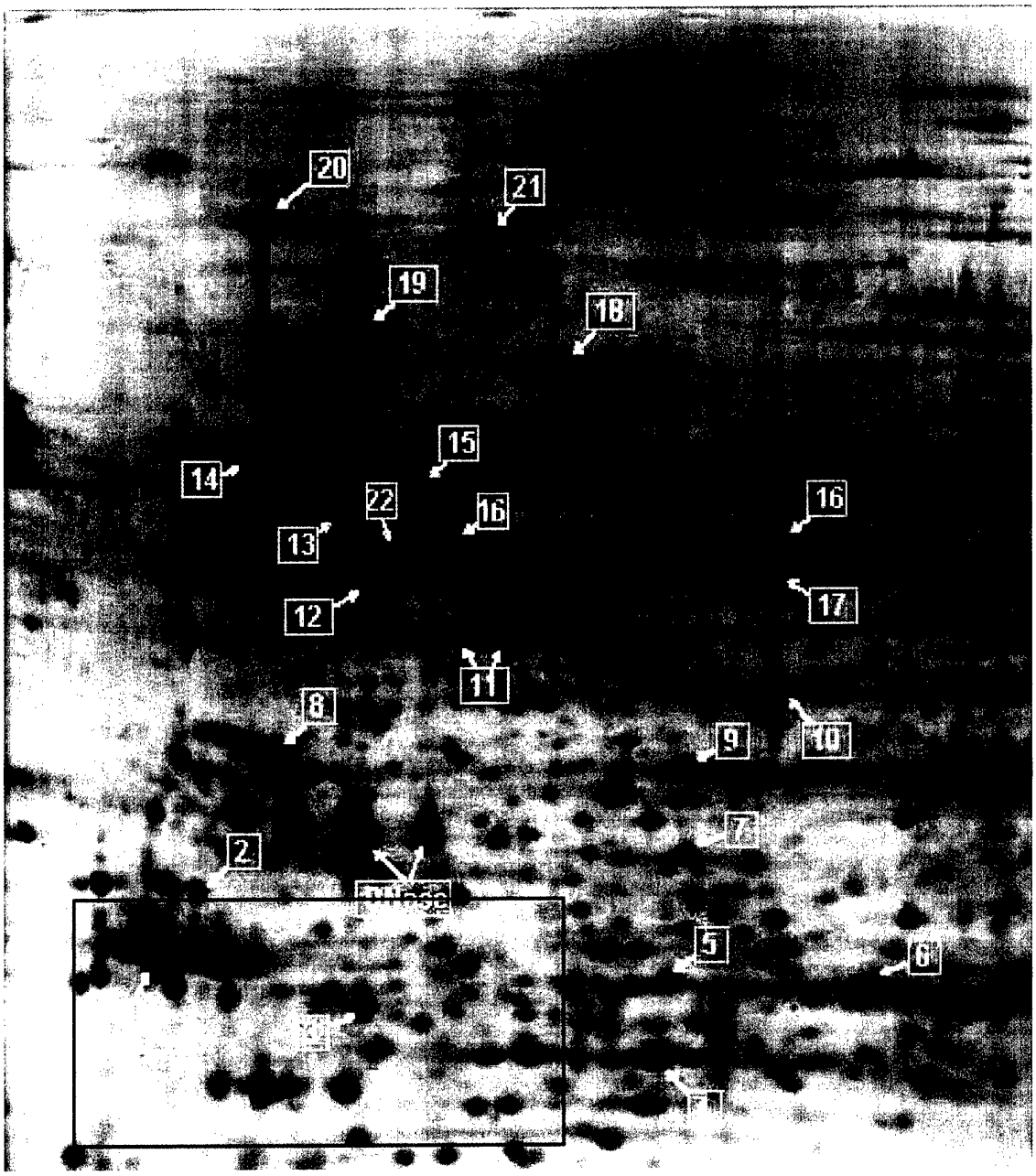


Figure 3.
Typical protein expression pattern of control cultured HEK. The horizontal axis is the isoelectric focusing dimension, with stretches for pH 4 (left) to pH 7 (right). The vertical axis is the SDS/PAGE dimension, which stretches from approximately 15 kDa (bottom) to about 120 kDa (top).
The numbers annotated on the gel refer to protein identities in Table 1. The area in the red box is shown in detail in Figure 4.

Spot #	Protein Name	Swiss Prot entry	Theor. MW (kDa)/pI	Matched peptide masses	Sequence coverage (%)	Z score
1.	14-3-3 σ (stratifin)	P31947	27.8/4.7	8	52	2.2
2.	tropomyosin α 3 chain, splice isoform 2	P06753	29.0/4.7	12	34	1.1
3.	ρ GDP-dissociation inhibitor 1	P52565	23.2/5.0	5	34	2.4
4.	glutathione-S-transferase P, α - chain	P09211	23.2/5.4	9	50	2.3
5.	heat shock protein 27, phosphorylated form	P04792	22.8/5.6	7	43	1.6
6.	heat shock protein 27, non-phosphorylated form	P04792	22.8/6.0	4	21	0.5
7.	annexin III	P12429	36.4/5.6	8	33	2.0
8.	nucleophosmin	P06748	32.6/4.6	4	15	0.5
9.	60S acidic ribosomal protein P0	P05388	34.3/5.7	7	34	2.1
10.	maspin	P36952	42.1/5.7	9	30	0.6
11.	β actin	P02570	41.7/5.3	8	31	2.2
12.	keratin 16	P08779	51.1/5.0	23	55	2.4
13.	β1 tubulin β2 tubulin	P07437 P05217	49.8/4.8 49.8/4.8	10 22	18 44	2.3 2.4
14.	protein disulfide isomerase, processed form	P07237	55.3/4.7	7	21	1.7
15.	α tubulin	P05209	49.9/4.9	4	12	0.5
16.	keratin 14	P02533	51.5/5.1	9	28	1.5
17.	keratin 17	Q04695	51.1/5.0	8	29	1.4
18.	heat shock cognate 71	P11142	70.8/5.4	15	27	2.0
19.	glucose related protein 78	P11021	72.3/5.1	14	23	2.3
20.	endoplasmin (grp94)	P14625	92.5/4.8	7	8	1.2
21.	transitional endoplasmic reticulum ATPase	P55072	89.3/5.1	13	17	2.0
22.	ATP synthase β chain, processed form	P06576	51.8/5.0	13	40	2.4

Table 1.
Keratinocyte proteins identified by MALDI-TOF/MS analysis. Numbers correspond to spots in Figure 3.
Values for MW and pI are according to the SWISSPROT database. For each protein the number of matched peptides, the % of sequence coverage and an indication for the reliability of the found protein (Z-score) are listed.

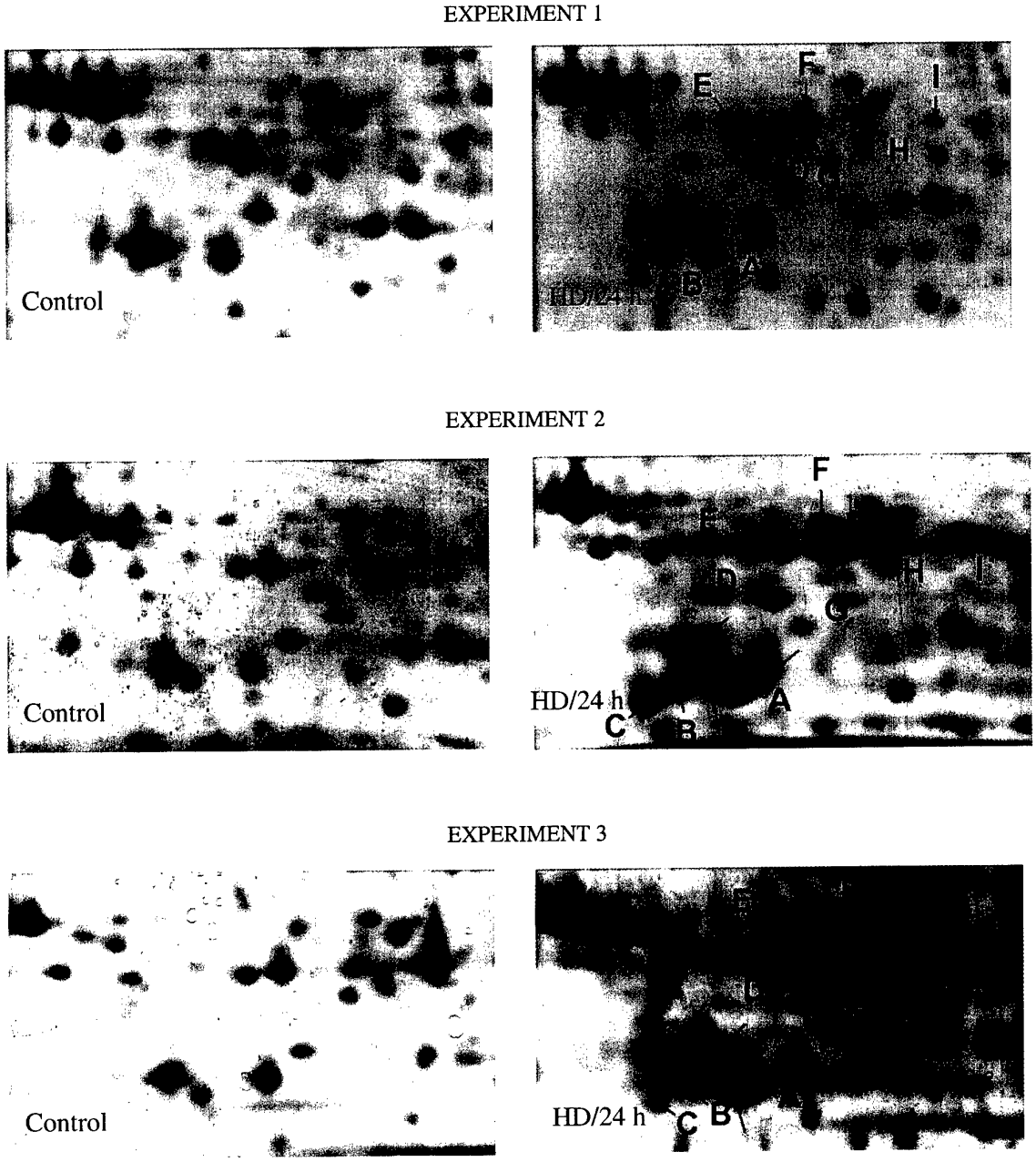


Figure 4.
Magnified view of the area indicated in Figure 3. Comparison of protein patterns in lysates of control HEK and HD-treated HEK, collected at 24 h after exposure to 100 μM HD. Typical gel patterns for each sample obtained in three independent experiments are given. For each pair of control and HD-treated gels, the difference spots are indicated with arrows. Difference spots which occurred in all three 24 h lysates are indicated with A through I and are considered to be treatment-dependent alterations in the protein profile.

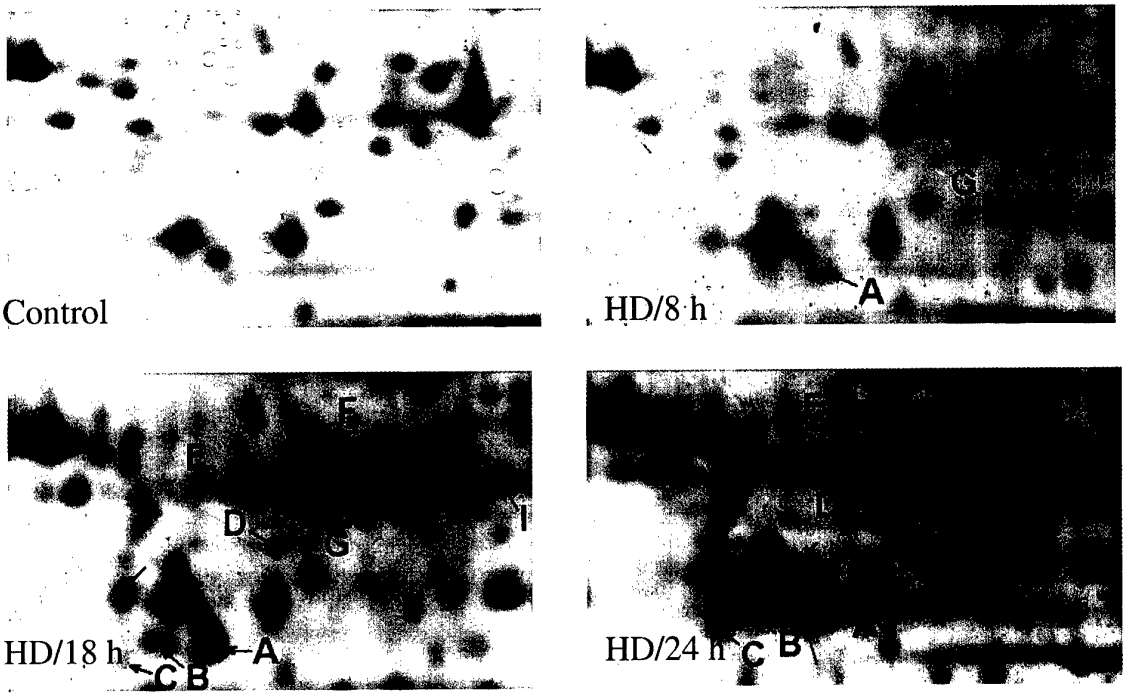


Figure 5.
Magnified view of the region indicated in Figure 3. Protein expression profiles are shown of HEK obtained at 8, 18 and 24 h after exposure to 100 μM HD and of unexposed HEK. The difference spots are indicated by arrows. Spots annotated with A through I represent proteins that are considered to be HD-exposure related (see legend of Figure 4).

TTCSR QFTSSSSMK **GSCGIGGGIGGGSSR ISSVLAGGSCR APSTYGGGLSVSSSR**
FSSGGAYGLGGGYGGGFSSSSSSFGSGFGGGYGGGLGAGLGGGFGGGFAGGDGLL
VGSEK VTMQLNDR LASYLDK VR **ALEEANADLEVK** IR DWYQR QRPAEIK
DYSPYFK **TIEDLR** NK **ILTATVDNANVLLQIDNAR LAADDFR** TK YETELNLR
MSVEADINGLR R **VLDELTLAR** ADLEMQIESLK EELAYLK K NHEEEMNALR
GQVGGDVNVEMDAAPGVDLSR ILNEMR DQYEK MAEK NR K DAEEWFFTK
TEELNR **EVATNSELVQSGK** SEISELR R TMQNLEIELQSQLSMK **ASLENSLEETK**
GR YCMQLAQIQEMIGSVEEQLAQLR CEMEQQNQEYK ILLDVK TR **LEQEIATYR** R
LLEGEDAHLSSSQFSSGSQSSR DVTSSSR QIR TK VMDVHDGK VVSTHEQVLR TK N

Figure 6a. The amino acid sequence of keratin 14. Indicated in red are the tryptic fragments that are found in spot G (Figure 4). Indicated in blue are the tryptic fragments that are found in spot A (Figure 4). The caspase motif VEMD is represented in green.

TTSIR QFTSSSSIK GSSGLGGGSSR TSCR LSGGLGAGSCR
LGSAGGLGSTLGGSSYSSCYSFGSGGGYGSSFGGVDGLLAGGEK ATMQNLNDR
LASYLDK VR ALEEANTELEVK IR DWYQR QAPGPAR DYSQYYR TIEELQNK
ILTATVDNANILLQIDNAR LAADDFR TK FETEQALR **LSVEADINGLR** R
VLDELTLAR ADLEMQIENLK EELAYLK K NHEEEMNALR
GQVGGEINVEMDAAPGVDLSR ILNEMR DQYEKMAEKNR K **DAEDWFFSK**
TEELNR EVATNSELVQSGK SEISELR R TMQALEIELQSQLSMK ASLEGNLAETEN
R YCVQLSQIQGLIGSVEEQLAQLR CEMEQQNQEYK ILLDVK **TR LEQEIATYR R**
LLEGEDAHLTQYK K EPVTTRQVR TIVEEVQDGK VISSR EQVHQTTR

Figure 6b. The amino acid sequence of keratin 17. Indicated in blue are the tryptic fragments that are found in spot C (Figure 4). The caspase motif VEMD is represented in green. One tryptic fragment is located before the VEMD motif.

4 Discussion

4.1 BB94

It is presumed that following exposure of skin to HD the balance is disrupted between basement membrane protein synthesis by keratinocytes and their degradation by MMPs. The result of this disturbance is loss of adherence between epidermis and dermis due to more net protein degradation than synthesis. One of the possibilities to restore this balance is to diminish or stop protein degradation by MMPs. BB94 is a hydroxamate-based broad-spectrum MMP inhibitor (Skotnicki et al., 1999; Yamamoto et al., 1998). The results of a previously reported study (Mol, 2000) encouraged further investigation of the effects of BB94 on the attenuation of epidermal-dermal separation. In the present study it is observed that 1.0 μg/ml BB94 is the lowest concentration of inhibitor that can be used to prevent HD-induced epidermal-dermal separation in organ-cultured human skin pieces. Furthermore, it is shown that application of 1.0 μg/ml BB94 to the culture medium is effective as late as 8 h after exposure, which means that the main factors leading to epidermal-dermal separation are not occurring before at least 8 h after exposure. This delay time might be even longer, as in the current experiment addition of BB94 at 8 h after exposure was the latest time point tested. Longer waiting times will be tested in the near future.

From a previous study (Mol, 2000) it was concluded that after exposure of human skin to HD the expression of MMP-1, -2, -3 and -9 did not noticeably exceed that in control skin, suggesting that there will be no HD-induced enhancement of proteolytic degradation. Together with the present observation, this tends towards the assumption that in time a deficiency arises of hemidesmosomal adhesion molecules, due to impaired synthesis or subsequent intracellular transport in keratinocytes. Inhibition of protein synthesis in keratinocytes is a well observed effect of HD (Mol and de Vries-van de Ruit, 1992). More detailed studies aimed to investigate the availability of proteins involved in the maintenance of the epidermal-dermal junction will be performed later on during this project, conform Task 4 described in the proposal.

4.2 Differential display of HEK proteins associated with exposure to HD

To identify proteins that are involved in the cellular response of HEK to HD exposure a proteomics approach was chosen, using 2D-PAGE to resolve the proteins and MS analysis to identify the protein spots. The benefit of this method is that the fate of proteins involved in the cellular response to HD could be studied on a wide scale. In the present study the window for proteins to be studied was chosen within the pI range 4-7 and the MW range 15-200 kDa. An important prerequisite for establishing reliable proteomic analysis is the development of suitable and reproducible methods for the separation and visualization of proteins by 2-D gel electrophoresis. We have paid attention to the selection of suitable protocols for lysate preparation and the protocols used here appeared to be the most practical. In order to obtain reproducible results, at least 4 replicate gels per sample were prepared. We observed rather often intergel differences for staining intensity of the same spot on replicate gels. This means that observation of differences in the spot patterns of the lysates can only be done in a qualitative manner. This is inherent to the applied silver staining, which is a sensitive protein stain but not quite suitable for detecting quantitative expression differences. In addition, the so-called "dynamic range" of response of the silver staining, i.e. the range in which the staining intensity increases with the amount of protein, is narrow. However, although no quantification was possible, reproducible appearance of spots in the pattern of HD-treated HEK in contrast to control HEK could be observed.

Following exposure of HEK to 100 μM HD, several new spots appeared in the protein profile at 24 h after exposure. Most of them were clustered in a distinct area of the gel, indicated with the red box in *Figure 1*. Identification by MS revealed two spots to be probably proteolytic fragments of K14 and one spot might be the C-terminal fragment of K17. Cleavage of other type I keratins (K15, K17, K18 and K19) as part of cytoskeletal remodeling during apoptosis has previously been demonstrated (Caulin et al., 1997; Badock et al., 2001; Ku et al., 1997; Prasad et al., 1998). This proteolysis is mediated by caspase-3 or caspase-6 activity on cleavage sites with motifs of VEVD or VEMD. The time-dependent appearance of fragments of K14 and K17 in the current experiments seems also to result from caspase-mediated proteolytic activity since the VEMD motif is present in both proteins. Since caspase activation is strongly involved in apoptosis, it is concluded that apoptosis takes place in cultured HEK that were exposed to 100 μM HD. These results are in agreement with previously reported findings that exposure of cells to HD causes apoptotic features (Rosenthal et al., 1998; Rosenthal et al., 2000). This observed involvement of apoptosis as a cellular response to HD insult, suggests that inhibition of apoptosis could be one of the concepts for specific therapeutic measures.

Identification of the remaining spots that appeared in the particular area of the gel following exposure of HEK to 100 μM HD is under way. To obtain a better resolution and separation of the protein spots in the area of interest, IEF will be performed over a narrow range of pI 4.5 -5.5. Additionally, to confirm the involvement of caspases in the fragmentation of keratins, a caspase inhibitor will be added to the post-exposure incubation medium.

5 Key research accomplishments

- MMP inhibitor BB94, when present in culture medium at a concentration of at least 1 μg/ml, is effective to prevent microvesication in *ex vivo* human skin pieces that were exposed for 5 min to saturated vapor of HD.
- BB94 can be added to the culture medium as late as 8 h after exposure to HD, without loosing its inhibiting effect on epidermal-dermal separation.
- On the 2-D proteome map of HEK, with MW range of 15-200 kDa and pI range of 4-7, 22 proteins were identified using MALDI –TOF MS peptide mass fingerprinting.
- Comparison of patterns obtained from control and HD-treated HEK revealed nine HD-exposure related changes in protein expression at 24 h after exposure. New spots appear with a gradually increase in intensity from 8 h following exposure.
- Three out of nine difference spots could be identified as fragments of keratin 14 and keratin 17, probably resulting from cleavage by caspase-6 at the VEMD motif.
- Exposure of cultured HEK to 100μM HD probably induces apoptosis.
- Application of proteomic analysis as an unbiased way to study cellular events following HD-exposure has proven to be a valuable approach

6 Reportable outcome:

M.A.E. Mol, C. van Dijk, E.S. Milder-Enacache, A.L. de Jong, A. Hulst, and B. van Baar (2002). Proteomics as a strategy to study the mechanistic toxicology of sulfur mustard. *Proceedings of the 2002 Medical Defense Bioscience Review, Baltimore MD.*

7 Conclusions

The first year of the research performed under Award Number DAMD17-02-1-0206 has yielded promising results with respect to the application of MMP inhibitor BB94 on HD-exposed human skin pieces in order to prevent microvesication. The observation that BB94 can be applied at least till 8 h after exposure without loosing its protecting effect supports the assumption that the cause of detachment of epidermis and dermis lies in a slowly growing deficiency of keratinocytes to provide new proteins for the hemidesmosomal attachment complex. From a therapeutic angle, this time window might allow non-urgent cure of HD casualties.

The use of 2D-PAGE analysis on the proteome of cultured HEK that were exposed to 100 μM HD gave clues for the induction of apoptosis by the gradual appearance over 24 h of fragments of the type I keratins 14 and 17. Also, several other new spots appeared in the protein pattern of HD-exposed HEK and their identification might shed more light on the mechanistic toxicology of HD.

The results obtained in this study so far, suggest a dual treatment of HD-exposed skin: application of a MMP-inhibitor to prevent microvesication together with the use of a caspase-inhibitor to reduce epidermal cell damage.